

**ENDOGLUCANASES****Field of the Invention**

This invention relates to newly identified polynucleotides, polypeptides encoded by such polynucleotides, the use of such polynucleotides and polypeptides, as well as the production and isolation of such polynucleotides and polypeptides. More particularly, the polypeptides of the present invention have been identified as endoglucanases and in particular, enzymes having carboxymethyl cellulase activity.

**Background**

Cellulose, a fibrous, tough, water-insoluble substance is found in the cell walls of plants, particularly, in stalks, stems, trunks and all the woody portions of plant tissues. Cellulose constitutes much of the mass of wood, and cotton is almost pure cellulose. Because cellulose is a linear, unbranched homopolysaccharide of 10,000 to 15,000 D-glucose units, it resembles amylose and the main chains of glycogen. But there is a very important difference, in cellulose, the glucose residues have the beta configuration, whereas in amylose, amylopectin and glycogen, the glucose is in the alpha configuration. The glucose residues in cellulose are linked by (beta 1→4) glycosidic bonds. This difference gives cellulose and amylose very different 3-dimensional structures and physical properties.

Cellulose cannot be used by most animals as a source of stored fuel, because the (beta 1→4) linkages of cellulose are not hydrolyzed by alpha-amylases. Termites readily digest cellulose but only because their intestinal tract harbors a symbiotic microorganism, *trichonympha*, which secretes cellulase, an enzyme that hydrolyses (beta 1→4) linkages between glucose units. The only vertebrates able to use cellulose as food are cattle and other ruminant animals (sheep, goats, camels and giraffes). The extra stomachs "rumens" of these animals teem with bacteria and protists that secrete cellulase.

The enzymatic hydrolysis of cellulose is considered to require the action of both endoglucanases (1,4-beta-D-glucan glucanohydrolase) and exoglucanases (1,4-beta-D-glucan cellobiohydrolase). A synergistic interaction of these enzymes is necessary for the complete hydrolysis of crystalline cellulose. (Caughlin, M.P., Genet. Eng. Rev., 3:39-109 (1985)). For the complete degradation of cellulose (cellulose to glucose),  $\beta$ -glucosidase might be required if the "exo" enzyme does not release glucose. 1,4- $\beta$ -D-glucan glucohydrolase is another type of "exo" cellulase.

Thermophilic bacteria have received considerable attention as sources of highly active and thermostable cellulolytic and xylanolytic enzymes (Bronneomeier, K. and Staudenbauer, W.L., D.R. Woods (Ed.), The Clostridia and Biotechnology, Butterworth Publishers, Stoneham, MA (1993). Recently, the most extremely thermophilic organotrophic eubacteria presently known have been isolated and characterized. These bacteria, which belong to the genus *thermotoga*, are fermentative microorganisms metabolizing a variety of carbohydrates (Huber, R. and Stetter, K.O., in Ballows, et al., (Ed.), The Procaryotes, 2nd Ed., Springer-Verlag, New York, pgs. 3809-3819 (1992)).

In Huber *et al.*, 1986, Arch. Microbiol. 144:324-333, the isolation of the bacterium *Thermotoga maritima* is described. *T. maritima* is a eubacterium that is strictly anaerobic, rod-shaped, fermentative, hyperthermophilic, and grows between 55°C and 90°C, with an optimum growth temperature of about 80°C. This eubacterium has been isolated from geothermally heated sea floors in Italy and the Azores. *T. maritima* cells have a sheath-like structure and monotrichous flagellation. *T. maritima* is classified in the eubacterium kingdom by virtue of having murein and fatty acid-containing lipids, diphtheria-toxin-resistant elongation factor 2, an RNA polymerase subunit pattern, and sensitivity to antibiotics.

Since, to date, most organisms identified from the archaeal domain are thermophiles or hyperthermophiles, archaeal bacteria are also considered a fertile source of thermophilic enzymes.

## Summary of the Invention

The present invention provides polynucleotides and polypeptides encoded thereby which have been identified as endoglucanase enzymes having carboxymethyl cellulase activity (CMC).

In accordance with one aspect of the present invention, there is provided novel enzymes, as well as active fragments, analogs and derivatives thereof.

In accordance with another aspect of the present invention, there are provided isolated nucleic acid molecules encoding enzymes of the present invention including mRNAs, DNAs, cDNAs, genomic DNAs as well as active analogs and fragments of such enzymes.

In accordance with another aspect of the present invention there are provided isolated nucleic acid molecules encoding mature polypeptides expressed by the DNA contained in ATCC Deposit No. 97516.

In accordance with yet a further aspect of the present invention, there is provided a process for producing such polypeptide by recombinant techniques comprising culturing recombinant prokaryotic and/or eukaryotic host cells, containing a nucleic acid sequence encoding an enzyme of the present invention, under conditions promoting expression of said enzyme and subsequent recovery of said enzyme.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such enzymes, or polynucleotide encoding such enzymes for degradation of cellulose for the conversion of plant biomass into fuels and chemicals, for use in detergents, the textile industry, in animal feed, in waste treatment, and in the fruit juice/brewing industry for the clarification and extraction of juices.

In accordance with yet a further aspect of the present invention, there is also provided nucleic acid probes comprising nucleic acid molecules of sufficient length to specifically hybridize to a nucleic acid sequence of the present invention.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such enzymes, or polynucleotides encoding such enzymes, for *in vitro* purposes related to scientific research, for example, to generate probes for identifying similar sequences which might encode similar enzymes from other organisms.

These and other aspects of the present invention should be apparent to those skilled in the art from the teachings herein.

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**BRIEF DESCRIPTION OF THE DRAWINGS**

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

Figures 1A-1X show the nucleotide and deduced amino acid sequences the enzymes of the present invention. Sequencing was performed using a 378 automated DNA sequencer (Applied Biosystems, Inc.).

## DETAILED DESCRIPTION OF THE INVENTION

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

A coding sequence is "operably linked to" another coding sequence when RNA polymerase will transcribe the two coding sequences into a single mRNA, which is then translated into a single polypeptide having amino acids derived from both coding sequences. The coding sequences need not be contiguous to one another so long as the expressed sequences are ultimately processed to produce the desired protein.

"Recombinant" enzymes refer to enzymes produced by recombinant DNA techniques; *i.e.*, produced from cells transformed by an exogenous DNA construct encoding the desired enzyme. "Synthetic" enzymes are those prepared by chemical synthesis.

A DNA "coding sequence of" or a "nucleotide sequence encoding" a particular enzyme, is a DNA sequence which is transcribed and translated into an enzyme when placed under the control of appropriate regulatory sequences. A "promotor sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. The promoter is part of the DNA sequence. This sequence region has a start codon at its 3' terminus. The promoter sequence does include the minimum number of bases where elements necessary to initiate transcription at levels detectable above background. However, after the RNA polymerase binds the sequence and transcription is initiated at the start codon (3' terminus with a promoter), transcription proceeds downstream in the 3' direction. Within the promotor sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1) as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

The present invention provides purified thermostable enzymes that catalyze the hydrolysis of the beta 1,4 glycosidic bonds in cellulose to thereby degrade cellulose. An exemplary purified enzyme is an endoglucanase derived from an organism referred herein as "AEPII1a" which is a thermophilic archaeal bacteria which has a very high temperature optimum. The organism is strictly anaerobic, rod-shaped and fermentative, and grows between 55 and 90°C (optimally at 85°C). AEPII1a was discovered in a shallow marine hydrothermal area in Vulcano, Italy. The organism has coccoid cells occurring in singlets or pairs. AEPII1a grows optimally at 85°C and pH 6.5 in a marine medium with cellulose as a substrate and nitrogen in gas phase. This exemplary enzyme is shown in Figure 1A, SEQ ID NO:2.

The polynucleotide encoding SEQ ID NO:2 was originally recovered from a genomic gene library derived from AEPII1a as described below. It contains an open reading frame encoding a protein of 553 amino acid residues.

In one embodiment, the endoglucanase enzyme of SEQ ID NO:2 of the present invention has a molecular weight of about 60.9 kilodaltons as measured by SDS-PAGE gel electrophoresis and an inferred molecular weight from the nucleotide sequence of the gene. This purified enzyme may be used to catalyze the enzymatic degradation of cellulose where desired. The endoglucanase enzyme of the present invention has a very high thermostability and has the closest homology to endo-1,4-beta-glucanase from *Xanthomonas campestris* with 50% identity and 71% similarity at the amino acid level.

In accordance with an aspect of the present invention, there are provided isolated nucleic acid molecules (polynucleotides) which encode for the mature enzymes having the deduced amino acid sequence of Figure 1A-X.

This invention, in addition to the isolated nucleic acid molecule encoding an endoglucanase enzyme disclosed in Figure 1 (SEQ ID NO:1), also provides substantially similar sequences. Isolated nucleic acid sequences are substantially similar if: (i) they are

capable of hybridizing under stringent conditions, hereinafter described, to SEQ ID NO:1; or (ii) they encode DNA sequences which are degenerate to SEQ ID NO:1. Degenerate DNA sequences encode the amino acid sequence of SEQ ID NO:2, but have variations in the nucleotide coding sequences. As used herein, "substantially similar" refers to the sequences having similar identity to the sequences of the instant invention. The nucleotide sequences that are substantially similar can be identified by hybridization or by sequence comparison. Enzyme sequences that are substantially similar can be identified by one or more of the following: proteolytic digestion, gel electrophoresis and/or microsequencing.

One means for isolating a nucleic acid molecule encoding an endoglucanase enzyme is to probe a genomic gene library with a natural or artificially designed probe using art recognized procedures (see, for example: Current Protocols in Molecular Biology, Ausubel F.M. *et al.* (EDS.) Green Publishing Company Assoc. and John Wiley Interscience, New York, 1989, 1992). It is appreciated to one skilled in the art that SEQ ID NO:1, or fragments thereof (comprising at least 15 contiguous nucleotides), is a particularly useful probe. Other particular useful probes for this purpose are hybridizable fragments to the sequences of SEQ ID NO:1 (*i.e.*, comprising at least 15 contiguous nucleotides).

With respect to nucleic acid sequences which hybridize to specific nucleic acid sequences disclosed herein, hybridization may be carried out under conditions of reduced stringency, medium stringency or even stringent conditions. As an example of oligonucleotide hybridization, a polymer membrane containing immobilized denatured nucleic acid is first prehybridized for 30 minutes at 45°C in a solution consisting of 0.9 M NaCl, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0, 5.0 mM Na<sub>2</sub>EDTA, 0.5% SDS, 10X Denhardt's, and 0.5 mg/mL polyriboadenylic acid. Approximately  $2 \times 10^7$  cpm (specific activity  $4-9 \times 10^6$  cpm/ug) of <sup>32</sup>P end-labeled oligonucleotide probe are then added to the solution. After 12-16 hours of incubation, the membrane is washed for 30 minutes at room temperature in 1X SET (150 mM NaCl, 20 mM Tris hydrochloride, pH 7.8, 1 mM Na<sub>2</sub>EDTA) containing 0.5% SDS, followed by a 30 minute wash in fresh 1X SET at T<sub>m</sub>-10°C for the oligo-nucleotide probe. The membrane is then exposed to auto-radiographic film for detection of hybridization signals.



Stringent conditions means hybridization will occur only if there is at least 90% identity, preferably at least 95% identity and most preferably at least 97% identity between the sequences. See J. Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (2d Ed. 1989) (Cold Spring Harbor Laboratory) which is hereby incorporated by reference in its entirety.

"Identity" as the term is used herein, refers to a polynucleotide sequence which comprises a percentage of the same bases as a reference polynucleotide (SEQ ID NO:1). For example, a polynucleotide which is at least 90% identical to a reference polynucleotide, has polynucleotide bases which are identical in 90% of the bases which make up the reference polynucleotide and may have different bases in 10% of the bases which comprise that polynucleotide sequence.

The present invention also relates to polynucleotides which differ from the reference polynucleotide such that the changes are silent changes, for example the changes do not alter the amino acid sequence encoded by the polynucleotide. The present invention also relates to nucleotide changes which result in amino acid substitutions, additions, deletions, fusions and truncations in the enzyme encoded by the reference polynucleotide (SEQ ID NO:1). In a preferred aspect of the invention these enzymes retain the same biological action as the enzyme encoded by the reference polynucleotide.

It is also appreciated that such probes can be and are preferably labeled with an analytically detectable reagent to facilitate identification of the probe. Useful reagents include but are not limited to radioactivity, fluorescent dyes or enzymes capable of catalyzing the formation of a detectable product. The probes are thus useful to isolate complementary copies of DNA from other animal sources or to screen such sources for related sequences.

The present invention provides substantially pure endoglucanase enzymes. The term "substantially pure" is used herein to describe a molecule, such as a polypeptide (*e.g.*, an

endoglucanase polypeptide, or a fragment thereof) that is substantially free of other proteins, lipids, carbohydrates, nucleic acids, and other biological materials with which it is naturally associated. For example, a substantially pure molecule, such as a polypeptide, can be at least 60%, by dry weight, the molecule of interest. The purity of the polypeptides can be determined using standard methods including, *e.g.*, polyacrylamide gel electrophoresis (*e.g.*, SDS-PAGE), column chromatography (*e.g.*, high performance liquid chromatography (HPLC)), and amino-terminal amino acid sequence analysis.

Endoglucanase polypeptides included in the invention can have one of the amino acid sequences of Endoglucanases shown in Figures 1A through 1X (SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 36, 38, 40, 42, 44, 46, and 48), for example, the amino acid sequence of AEPII1a (SEQ ID NO:2). Endoglucanase polypeptides, such as those isolated from AEPII1a, can be characterized by catalyzing the hydrolysis of the beta 1,4 glycosidic bonds in cellulose.

Also included in the invention are polypeptides having sequences that are "substantially identical" to the sequence of an endoglucanase polypeptide, such as one of SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 36, 38, 40, 42, 44, 46, and 48, *e.g.*, SEQ ID NO:2. A "substantially identical" amino acid sequence is a sequence that differs from a reference sequence only by conservative amino acid substitutions, for example, substitutions of one amino acid for another of the same class (*e.g.*, substitution of one hydrophobic amino acid, such as isoleucine, valine, leucine, or methionine, for another, or substitution of one polar amino acid for another, such as substitution of arginine for lysine, glutamic acid for aspartic acid, or glutamine for asparagine), or by one or more non-conservative substitutions, deletions, or insertions, provided that the polypeptide retains at least one endoglucanase-specific activity or an endoglucanase-specific epitope. For example, one or more amino acids can be deleted from an endoglucanase polypeptide, resulting in modification of the structure of the polypeptide, without significantly altering its biological activity. For example, amino- or carboxyl-terminal amino acids that are not required for

endoglucanase biological activity, can be removed. Such modifications can result in the development of smaller active endoglucanase polypeptides.

Other endoglucanase polypeptides included in the invention are polypeptides having amino acid sequences that are at least 50% identical to the amino acid sequence of an endoglucanase polypeptide, such as any of endoglucanases in SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 36, 38, 40, 42, 44, 46, and 48, *e.g.*, SEQ ID NO:2. The length of comparison in determining amino acid sequence homology can be, for example, at least 15 amino acids, for example, at least 20, 25, or 35 amino acids. Homology can be measured using standard sequence analysis software (*e.g.*, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705; also see Ausubel, *et al.*, *supra*).

The invention also includes fragments of endoglucanase polypeptides that retain at least one endoglucanase-specific activity or epitope. Endoglucanase activity can be assayed by examining the catalysis of beta 1,4 glycosidic bonds in cellulose. For example, an endoglucanase polypeptide fragment containing, *e.g.*, at least 8-10 amino acids can be used as an immunogen in the production of endoglucanase-specific antibodies. The fragment can contain, for example, an amino acid sequence that is conserved in endoglucanases, and this amino acid sequence can contain amino acids that are conserved in endoglucanases. Such fragments can easily be identified by comparing the sequences of endoglucanases found in Figures 1A-1X. In addition to their use as peptide immunogens, the above-described endoglucanase fragments can be used in immunoassays, such as ELISAs, to detect the presence of endoglucanase-specific antibodies in samples.

The endoglucanase polypeptides of the invention can be obtained using any of several standard methods. For example, endoglucanase polypeptides can be produced in a standard recombinant expression systems (see below), chemically synthesized (this approach may be

limited to small endoglucanase peptide fragments), or purified from organisms in which they are naturally expressed.

The invention also provides isolated nucleic acid molecules that encode the endoglucanase polypeptides described above, as well as fragments thereof. For example, nucleic acids that encode any of SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 36, 38, 40, 42, 44, 46, and 48, are included in the invention. These nucleic acids can contain naturally occurring nucleotide sequences, or sequences that differ from those of the naturally occurring nucleic acids that encode endoglucanases, but encode the same amino acids, due to the degeneracy of the genetic code. The nucleic acids of the invention can contain DNA or RNA nucleotides, or combinations or modifications thereof. Exemplary nucleic acids of the invention are shown in SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, and 47.

By "isolated nucleic acid" is meant a nucleic acid, *e.g.*, a DNA or RNA molecule, that is not immediately contiguous with the 5' and 3' flanking sequences with which it normally is immediately contiguous when present in the naturally occurring genome of the organism from which it is derived. The term thus describes, for example, a nucleic acid that is incorporated into a vector, such as a plasmid or viral vector; a nucleic acid that is incorporated into the genome of a heterologous cell (or the genome of a homologous cell, but at a site different from that at which it naturally occurs); and a nucleic acid that exists as a separate molecule, *e.g.*, a DNA fragment produced by PCR amplification or restriction enzyme digestion, or an RNA molecule produced by *in vitro* transcription. The term also describes a recombinant nucleic acid that forms part of a hybrid gene encoding additional polypeptide sequences that can be used, for example, in the production of a fusion protein.

The nucleic acid molecules of the invention can be used as templates in standard methods for production of endoglucanase gene products (*e.g.*, endoglucanase RNAs and endoglucanase polypeptides). In addition, the nucleic acid molecules that encode

endoglucanase polypeptides (and fragments thereof) and related nucleic acids, such as (1) nucleic acids containing sequences that are complementary to, or that hybridize to, nucleic acids encoding endoglucanase polypeptides, or fragments thereof (*e.g.*, fragments containing at least 12, 15, 20, or 25 nucleotides); and (2) nucleic acids containing sequences that hybridize to sequences that are complementary to nucleic acids encoding endoglucanase polypeptides, or fragments thereof (*e.g.*, fragments containing at least 12, 15, 20, or 25 nucleotides); can be used in methods focused on their hybridization properties. For example, as is described in further detail below, such nucleic acid molecules can be used in the following methods: PCR methods for synthesizing endoglucanase nucleic acids, methods for detecting the presence of an endoglucanase nucleic acid in a sample, screening methods for identifying nucleic acids encoding new endoglucanase family members.

The invention also includes methods for identifying nucleic acid molecules that encode members of the endoglucanase polypeptide family in addition to SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 36, 38, 40, 42, 44, 46, and 48. In these methods, a sample, *e.g.*, a nucleic acid library, such as a cDNA library, that contains a nucleic acid encoding an endoglucanase polypeptide is screened with an endoglucanase-specific probe, *e.g.*, an endoglucanase-specific nucleic acid probe. Endoglucanase-specific nucleic acid probes are nucleic acid molecules (*e.g.*, molecules containing DNA or RNA nucleotides, or combinations or modifications thereof) that specifically hybridize to nucleic acids encoding endoglucanase polypeptides, or to complementary sequences thereof. The term "endoglucanase-specific probe," in the context of this method of invention, refers to probes that bind to nucleic acids encoding endoglucanase polypeptides, or to complementary sequences thereof, to a detectably greater extent than to nucleic acids encoding other enzymes, or to complementary sequences thereof.

The invention facilitates production of endoglucanase-specific nucleic acid probes. Methods for obtaining such probes can be designed based on the amino acid sequences shown in Figure 1. The probes, which can contain at least 12, *e.g.*, at least 15, 25, 35, 50, 100, or 150 nucleotides, can be produced using any of several standard methods (see, *e.g.*, Ausubel,

*et al., supra*). For example, preferably, the probes are generated using PCR amplification methods. In these methods, primers are designed that correspond to endoglucanase-conserved sequences (see Figure 1), which can include endoglucanase-specific amino acids, and the resulting PCR product is used as a probe to screen a nucleic acid library, such as a cDNA library.

In accordance with another aspect of the present invention, there is provided an isolated polynucleotide encoding an exemplary enzyme of the present invention (SEQ ID NO:1) which has been deposited with an appropriate depository for the deposit of biological material. The deposited material is a pQET (Qiagen, Inc.) plasmid comprising the DNA of Figure 1A. The deposit has been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, USA, on April 22, 1996 and assigned ATCC Deposit No. 97516.

The deposit has been made under the terms of the Budapest Treaty on the International Recognition of the deposit of micro-organisms for purposes of patent procedure. The strain will be irrevocably and without restriction or condition released to the public upon the issuance of a patent. The deposit is provided merely as convenience to those of skill in the art and are not an admission that a deposit be required under 35 U.S.C. §112. The sequences of the polynucleotides contained in the deposited materials, as well as the amino acid sequences of the polypeptides encoded thereby, are controlling in the event of any conflict with any description of sequences herein. A license may be required to make, use or sell the deposited materials, and no such license is hereby granted.

The coding sequences for the endoglucanase enzymes of the present invention were identified by preparing an AEPII1a genomic DNA library, for example, and screening the library for the clones having endoglucanase activity. Such methods for constructing a genomic gene library are well-known in the art. One means, for example, comprises shearing DNA isolated from AEPII1a by physical disruption. A small amount of the sheared DNA is checked on an agarose gel to verify that the majority of the DNA is in the desired size range

(approximately 3-6 kb). The DNA is then blunt ended using Mung Bean Nuclease, incubated at 37°C and phenol/chloroform extracted. The DNA is then methylated using Eco RI Methylase. Eco RI linkers are then ligated to the blunt ends through the use of T4 DNA ligase and incubation at 4°C. The ligation reaction is then terminated and the DNA is cut-back with Eco RI restriction enzyme. The DNA is then size fractionated on a sucrose gradient following procedures known in the art, for example, Maniatis, T., *et al.*, Molecular Cloning, Cold Spring Harbor Press, New York, 1982, which is hereby incorporated by reference in its entirety.

A plate assay is then performed to get an approximate concentration of the DNA. Ligation reactions are then performed and 1 µl of the ligation reaction is packaged to construct a library. Packaging, for example, may occur through the use of purified λgt11 phage arms cut with EcoRI and DNA cut with EcoRI after attaching EcoRI linkers. The DNA and λgt11 arms are ligated with DNA ligase. The ligated DNA is then packaged into infectious phage particles. The packaged phages are used to infect E. coli cultures and the infected cells are spread on agar plates to yield plates carrying thousands of individual phage plaques. The library is then amplified.

In a preferred embodiment, the enzyme of the present invention, was isolated from an AEPIIIa library by the following technique:

1.  $\lambda$ gt11 AEPIIIa library was plated onto 6 LB/GelRite/0.1% CMC/NZY agar plates (~4,800 plaque forming units/plate) in E.coli Y1090 host with LB agarose containing 1mM IPTG as top agarose. The plates were incubated at 37°C overnight.
2. Plates were chilled at 4°C for one hour.
3. The plates were overlayed with Duralon membranes (Stratagene) at room temperature for one hour and the membranes were oriented and lifted off the plates and stored at 4°C.
4. The top agarose layer was removed and plates were incubated at 72°C for ~3 hours.
5. The plate surface was rinsed with NaCl.
6. The plate was stained with 0.1% Congo Red for 15 minutes.
7. The plate was destained with 1M NaCl.
8. The putative positives identified on plate were isolated from the Duralon membrane (positives are identified by clearing zones around clones). The phage was eluted from the membrane by incubating in 500 $\mu$ l SM + 25 $\mu$ l CHCl<sub>3</sub> to elute.
9. Insert DNA was subcloned into pBluescript II SK(±) cloning vector (Stratagene), and subclones were reassayed for CMCase activity using the following protocol:
  - i) Spin 1ml overnight miniprep of clone at maximum speed for 3 minutes.
  - ii) Decant the supernatant and use it to fill "wells" that have been made in an LB/GelRite/0.1% CMC plate.
  - iii) Incubate at 72°C for 2 hours.
  - iv) Stain with 0.1% Congo Red for 15 minutes.
  - v) Destain with 1M NaCl for 15 minutes.
  - vi) Identify positives by clearing zone around clone.

Fragments of the full length gene of the present invention may be used as a hybridization probe for a cDNA or a genomic library to isolate the full length DNA and to isolate other DNAs which have a high sequence similarity to the gene or similar biological



activity. Probes of this type have at least 10, preferably at least 15, and even more preferably at least 30 bases and may contain, for example, at least 50 or more bases. The probe may also be used to identify a DNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete gene including regulatory and promotor regions, exons, and introns.

The isolated nucleic acid sequences and other enzymes may then be measured for retention of biological activity characteristic to the enzyme of the present invention, for example, in an assay for detecting enzymatic endoglucanase activity. Such enzymes include truncated forms of endoglucanase, and variants such as deletion and insertion variants.

Examples of such assays include an assay for the detection of endoglucanase activity based on specific interaction of direct dyes such as Congo red with polysaccharides. This colorant reacts with beta-1,4-glucans causing a visible red shift (Wood, P.J., Carbohydr. Res., 85:271 (1980) and Wood, P.J., Carbohydr. Res., 94:c19 (1981)). The preferred substrate for the test is carboxymethylcellulose (CMC) which can be obtained from different sources (Hercules Inc., Wilmington, DE, Type 4M6F or Sigma Chemical Company, St. Louis, MO, Medium Viscosity). The CMC is incorporated as the main carbon sources into a minimal agar medium in quantities of 0.1-1.0%. The microorganisms can be screened directly on these plates, but the replica plating technique from a master plate is preferable since the visualization of the activity requires successive flooding with the reagents, which would render the reisolation of active colonies difficult. Such endoglucanase-producing colonies are detectable after a suitable incubation time (1-3 days depending on the growth), by flooding the plate with 10 ml of a 0.1% aqueous solution of Congo Red. The coloration is terminated after 20 minutes by pouring off the dye and flooding the plates with 10 ml of 5M NaCl solution (commercial salt can be used). After an additional 20 minutes, the salt solution is discarded and endoglucanase activity is revealed by a pale-orange zone around the active microorganisms. In some cases, these zones can be enhanced by treating the plates with 1 N acetic acid, causing the dye to change its color to blue.

The same technique can be used as a cup-plate diffusion assay with excellent sensitivity for the determination of endoglucanase activity in culture filtrates or during enzyme purification steps (Carger, J.H., Anal. Biochem., 153:75 (1986)). See generally, Methods for Measuring Cellulase Activities, Methods in Enzymology, Vol. 160, pgs. 87-116.

The enzyme of the present invention has enzymatic activity with respect to the hydrolysis of the beta 1,4 glycosidic bonds in carboxymethylcellulose, since the halos discussed above are shown around the colonies.

The polynucleotide of the present invention may be in the form of DNA which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature enzyme may be identical to the coding sequences shown in Figure 1 and/or that of the deposited clone (SEQ ID NO:1), or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same mature enzyme as the DNA of Figure 1 (*e.g.*, SEQ ID NO:1).

The polynucleotide which encodes for the mature enzyme of Figure 1 (*e.g.*, SEQ ID NO:2) may include, but is not limited to: only the coding sequence for the mature enzyme; the coding sequence for the mature enzyme and additional coding sequence such as a leader sequence or a proprotein sequence; the coding sequence for the mature enzyme (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature enzyme.

Thus, the term "polynucleotide encoding an enzyme (protein)" encompasses a polynucleotide which includes only coding sequence for the enzyme as well as a polynucleotide which includes additional coding and/or non-coding sequence.

The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments, analogs and derivatives of the enzyme having the deduced amino acid sequence of Figure 1 (e.g., SEQ ID NO:2). The variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide.

Thus, the present invention includes polynucleotides encoding the same mature enzyme as shown in Figure 1 as well as variants of such polynucleotides which variants encode for a fragment, derivative or analog of the enzyme of Figure 1. Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As hereinabove indicated, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in Figure 1. As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which may have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded enzyme.

The present invention also includes polynucleotides, wherein the coding sequence for the mature enzyme may be fused in the same reading frame to a polynucleotide sequence which aids in expression and secretion of an enzyme from a host cell, for example, a leader sequence which functions to control transport of an enzyme from the cell. The enzyme having a leader sequence is a preprotein and may have the leader sequence cleaved by the host cell to form the mature form of the enzyme. The polynucleotides may also encode for a proprotein which is the mature protein plus additional 5' amino acid residues. A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.

Thus, for example, the polynucleotide of the present invention may encode for a mature enzyme, or for an enzyme having a prosequence or for an enzyme having both a prosequence and a presequence (leader sequence).

The present invention further relates to polynucleotides which hybridize to the hereinabove-described sequences if there is at least 70%, preferably at least 90%, and more preferably at least 95% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to the hereinabove-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotides in a preferred embodiment encode enzymes which either retain substantially the same biological function or activity as the mature enzyme encoded by the DNA of Figure 1.

Alternatively, the polynucleotide may have at least 15 bases, preferably at least 30 bases, and more preferably at least 50 bases which hybridize to a polynucleotide of the present invention and which has an identity thereto, as hereinabove described, and which may or may not retain activity. For example, such polynucleotides may be employed as probes for the polynucleotide of SEQ ID NO:1, for example, for recovery of the polynucleotide or as a PCR primer.

Thus, the present invention is directed to polynucleotides having at least a 70% identity, preferably at least 90% identity and more preferably at least a 95% identity to a polynucleotide which encodes the enzyme of SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 36, 38, 40, 42, 44, 46, or 48 as well as fragments thereof, which fragments have at least 30 bases and preferably at least 50 bases and to enzymes encoded by such polynucleotides.

The present invention further relates to a enzyme which has the deduced amino acid sequence of Figure 1, as well as fragments, analogs and derivatives of such enzyme.

The terms "fragment," "derivative" and "analog" when referring to the enzyme of Figure 1 means a enzyme which retains essentially the same biological function or activity as

such enzyme. Thus, an analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature enzyme.

The enzyme of the present invention may be a recombinant enzyme, a natural enzyme or a synthetic enzyme, preferably a recombinant enzyme.

The fragment, derivative or analog of the enzyme of Figure 1 may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature enzyme is fused with another compound, such as a compound to increase the half-life of the enzyme (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature enzyme, such as a leader or secretory sequence or a sequence which is employed for purification of the mature enzyme or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The enzymes and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or enzyme present in a living animal is not isolated, but the same polynucleotide or enzyme, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or enzymes could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The enzymes of the present invention include an enzyme of Figure 1A-1X (in particular the mature enzyme) as well as enzymes which have at least 70% similarity (preferably at least 70% identity) to an enzyme of Figure 1A-1X and more preferably at least 90% similarity (more preferably at least 90% identity) to an enzyme of Figure 1A-1X and still more preferably at least 95% similarity (still more preferably at least 95% identity) to an enzyme of Figure 1A-1X and also include portions of such enzymes with such portion of the enzyme generally containing at least 30 amino acids and more preferably at least 50 amino acids.

As known in the art "similarity" between two enzymes is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one enzyme to the sequence of a second enzyme. Similarity may be determined by procedures which are well-known in the art, for example, a BLAST program (Basic Local Alignment Search Tool at the National Center for Biological Information).

A variant, i.e. a "fragment", "analog" or "derivative" enzyme, and reference enzyme may differ in amino acid sequence by one or more substitutions, additions, deletions, fusions and truncations, which may be present in any combination.

Among preferred variants are those that vary from a reference by conservative amino acid substitutions. Such substitutions are those that substitute a given amino acid in a polypeptide by another amino acid of like characteristics. Typically seen as conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu and Ile; interchange of the hydroxyl residues Ser and Thr, exchange of the acidic residues Asp and Glu, substitution between the amide residues Asn and Gln, exchange of the basic residues Lys and Arg and replacements among the aromatic residues Phe, Tyr.

Most highly preferred are variants which retain the same biological function and activity as the reference polypeptide from which it varies.

Fragments or portions of the enzymes of the present invention may be employed for producing the corresponding full-length enzyme by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length enzymes. Fragments or portions of the polynucleotides of the present invention may be used to synthesize full-length polynucleotides of the present invention.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of enzymes of the invention by recombinant techniques.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors containing the polynucleotides of this invention. Such vectors may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the genes of the present invention. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotides of the present invention may be employed for producing enzymes by recombinant techniques. Thus, for example, the polynucleotide may be included in any one of a variety of expression vectors for expressing an enzyme. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction

endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the *E. coli*. *lac* or *trp*, the phage lambda P<sub>L</sub> promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in *E. coli*.

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein.

As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as *E. coli*, *Streptomyces*, *Bacillus subtilis*; fungal cells, such as yeast; insect cells such as *Drosophila S2* and *Spodoptera Sf9*; animal cells such as CHO, COS or Bowes melanoma; adenoviruses; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this



embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example; Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pBluescript II (Stratagene); pTRC99a, pKK223-3, pDR540, pRIT2T (Pharmacia); Eukaryotic: pXT1, pSG5 (Stratagene) pSVK3, pBPV, pMSG, pSVLSV40 (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P<sub>R</sub>, P<sub>L</sub> and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis, L., Dibner, M., Battey, I., Basic Methods in Molecular Biology, (1986)).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the enzymes of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the

present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989), the disclosure of which is hereby incorporated by reference.

Transcription of the DNA encoding the enzymes of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples include the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK),  $\alpha$ -factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated enzyme. Optionally, the heterologous sequence can encode a fusion enzyme including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium*

and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell-lysing agents, such methods are well known to those skilled in the art.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, *Cell*, 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

The enzyme can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The enzymes of the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the enzymes of the present invention may be glycosylated or may be non-glycosylated. Enzymes of the invention may or may not also include an initial methionine amino acid residue.

The enzyme of this invention may be employed for any purpose in which such enzyme activity is necessary or desired. In a preferred embodiment the enzyme is employed for catalyzing the hydrolysis of cellulose. The degradation of cellulose may be used for the conversion of plant biomass into fuels and chemicals.

The enzyme of the present invention may also be employed in the detergent and textile industry, in the production of animal feed, in waste treatment and in the fruit juice/brewing industry for the clarification and extraction of juices.

In a preferred embodiment, the enzyme of the present invention is a thermostable enzyme which is stable to heat and is heat resistant and catalyzes the enzymatic hydrolysis of cellulose, *i.e.*, the enzyme is able to renature and regain activity after a brief (*i.e.*, 5 to 30 seconds), or longer period, for example, minutes or hours, exposure to temperatures of 80°C to 105°C and has a temperature optimum above 60°C.

The enzymes, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain, and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

Antibodies generated against the enzymes corresponding to a sequence of the present invention can be obtained by direct injection of the enzymes into an animal or by administering the enzymes to an animal, preferably a nonhuman. The antibody so obtained will then bind the enzymes itself. In this manner, even a sequence encoding only a fragment of the enzymes can be used to generate antibodies binding the whole native enzymes. Such antibodies can then be used to isolate the enzyme from cells expressing that enzyme.

For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, 1975, *Nature*, 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, *Immunology Today* 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole, et al., 1985, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96).

Techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to immunogenic enzyme products of this invention. Also, transgenic mice may be used to express humanized antibodies to immunogenic enzyme products of this invention.

Antibodies generated against the enzyme of the present invention may be used in screening for similar enzymes from other organisms and samples. Such screening techniques are known in the art, for example, one such screening assay is described in "Methods for Measuring Cellulase Activities", *Methods in Enzymology*, Vol 160, pp. 87-116, which is

hereby incorporated by reference in its entirety. Antibodies may also be employed as a probe to screen gene libraries generated from this or other organisms to identify this or cross reactive activities.

Isolation and purification of polypeptides produced in the systems described above can be carried out using conventional methods, appropriate for the particular system. For example, preparative chromatography and immunological separations employing antibodies, such as monoclonal or polyclonal antibodies, can be used.

The term "antibody," as used herein, refers to intact immunoglobulin molecules, as well as fragments of immunoglobulin molecules, such as Fab, Fab', (Fab')<sub>2</sub>, Fv, and SCA fragments, that are capable of binding to an epitope of an endoglucanase polypeptide. These antibody fragments, which retain some ability to selectively bind to the antigen (*e.g.*, an endoglucanase antigen) of the antibody from which they are derived, can be made using well known methods in the art (see, *e.g.*, Harlow and Lane, *supra*), and are described further, as follows.

- (1) A Fab fragment consists of a monovalent antigen-binding fragment of an antibody molecule, and can be produced by digestion of a whole antibody molecule with the enzyme papain, to yield a fragment consisting of an intact light chain and a portion of a heavy chain.
- (2) A Fab' fragment of an antibody molecule can be obtained by treating a whole antibody molecule with pepsin, followed by reduction, to yield a molecule consisting of an intact light chain and a portion of a heavy chain. Two Fab' fragments are obtained per antibody molecule treated in this manner.
- (3) A (Fab')<sub>2</sub> fragment of an antibody can be obtained by treating a whole antibody molecule with the enzyme pepsin, without subsequent reduction. A (Fab')<sub>2</sub> fragment is a dimer of two Fab' fragments, held together by two disulfide bonds.

(4) An Fv fragment is defined as a genetically engineered fragment containing the variable region of a light chain and the variable region of a heavy chain expressed as two chains.

(5) A single chain antibody ("SCA") is a genetically engineered single chain molecule containing the variable region of a light chain and the variable region of a heavy chain, linked by a suitable, flexible polypeptide linker.

As used in this invention, the term "epitope" refers to an antigenic determinant on an antigen, such as an endoglucanase polypeptide, to which the paratope of an antibody, such as an endoglucanase-specific antibody, binds. Antigenic determinants usually consist of chemically active surface groupings of molecules, such as amino acids or sugar side chains, and can have specific three-dimensional structural characteristics, as well as specific charge characteristics.

As is mentioned above, antigens that can be used in producing endoglucanase-specific antibodies include endoglucanase polypeptides, *e.g.*, any of the endoglucanases shown in Figures 1A-1X polypeptide fragments. The polypeptide or peptide used to immunize an animal can be obtained by standard recombinant, chemical synthetic, or purification methods. As is well known in the art, in order to increase immunogenicity, an antigen can be conjugated to a carrier protein. Commonly used carriers include keyhole limpet hemocyanin (KLH), thyroglobulin, bovine serum albumin (BSA), and tetanus toxoid. The coupled peptide is then used to immunize the animal (*e.g.*, a mouse, a rat, or a rabbit). In addition to such carriers, well known adjuvants can be administered with the antigen to facilitate induction of a strong immune response.

Endoglucanase-specific polyclonal and monoclonal antibodies can be purified, for example, by binding to, and elution from, a matrix containing an endoglucanase polypeptide, *e.g.*, the endoglucanase polypeptide (or fragment thereof) to which the antibodies were raised. Additional methods for antibody purification and concentration are well known in the art and can be practiced with the endoglucanase-specific antibodies of the invention (see, for example, C-oligan, *et al.*, Unit 9, *Current Protocols in Immunology*, Wiley Interscience, 1994).

Anti-idiotypic antibodies corresponding to endoglucanase-specific antigens are also included in the invention, and can be produced using standard methods. These antibodies are raised to endoglucanase-specific antibodies, and thus mimic endoglucanase-specific epitopes.

The members of a pair of molecules (*e.g.*, an antibody-antigen pair or a nucleic acid pair) are said to "specifically bind" to each other if they bind to each other with greater affinity than to other, non-specific molecules. For example, an antibody raised against an antigen to which it binds more efficiently than to a non-specific protein can be described as specifically binding to the antigen. (Similarly, a nucleic acid probe can be described as specifically binding to a nucleic acid target if it forms a specific duplex with the target by base pairing interactions (see above).)

The present invention is further described with reference to the following examples; however, it is to be understood that the present invention is not limited to such examples. All parts or amounts, unless otherwise specified, are by weight.

In one aspect of the invention, a method for producing an endoglucanase enzyme, such as those shown in Figures 1A-1X, is provided. The method includes growing a host cell which contains a polynucleotide encoding the enzyme (*e.g.*, SEQ ID NO: NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, or 47), under conditions which allow the expression of the nucleic acid, and isolating the enzyme encoded by the nucleic acid. Methods of culturing the host cell are described in the Examples and are known by those of skill in the art.

In another embodiment, the invention provides a method for degrading carboxymethylcellulose. The method includes contacting carboxymethylcellulose with a degrading effective amount of an enzyme of the invention, such as the enzyme shown in SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 36, 38, 40, 42, 44, 46, or 48. The term "degrading effective" amount refers to the amount of enzyme which is required to degrade at least 50% of the carboxymethylcellulose, as compared to carboxymethylcellulose



not contacted with the enzyme. Preferably, at least 80% of the carboxymethylcellulose is degraded.

In another embodiment, the invention provides a method for hydrolyzing the beta 1,4 glycosidic bond in cellulose, the method including administering an effective amount of an enzyme of the invention (*e.g.*, SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 36, 38, 40, 42, 44, 46, or 48) to cellulose, to hydrolyze the glycosidic bond. An "effective" amount refers to the amount of enzyme which is required to hydrolyze at least 50% of the glycosidic bonds, as compared to carboxymethylcellulose not contacted with the enzyme. Preferably, at least 80% of the glycosidic bonds are hydrolyzed.

In order to facilitate understanding of the following examples certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1 µg of plasmid or DNA fragment is used with about 2 units of enzyme in about 20 µl of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 µg of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary

in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.

Size separation of the cleaved fragments is generally performed using 8 percent polyacrylamide gel described by Goeddel, D. *et al.*, Nucleic Acids Res., 8:4057 (1980), for example.

"Oligonucleotides" refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides may or may not have a 5' phosphate. Those that do not will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Maniatis, T., *et al.*, *Id.*, p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 µg of approximately equimolar amounts of the DNA fragments to be ligated.

Unless otherwise stated, transformation was performed as described in the method of Sambrook, Fritsch and Maniatus, 1989. The following examples are intended to illustrate, but not to limit, the invention. While the procedures described in the examples are typical of those that can be used to carry out certain aspects of the invention, other procedures known to those skilled in the art can also be used. The following materials and methods were used in carrying out the experiments described in the examples.

### Example 1

#### Bacterial Expression and Purification of Endoglucanase

An AEPIIIa genomic library was constructed in the Lambda gt11 cloning vector (Stratagene Cloning Systems). The library was screened in Y1090 *E. coli* cells (Stratagene) for endoglucanase activity and a positive clone was identified and isolated. DNA of this clone was used as a template in a 100 ul PCT reaction using the following primer sequences:

5' primer: AATAGCGGCCGCAAGCTTATCGACGGTTTCCATATGGGGATTGGTG (SEQ ID NO:49). 3' primer: AATAGCGGCCGCGGATCCAGACCAACTGG TAATGGTAGCGAC (SEQ ID NO:50).

The PCR reaction product was purified and digested with Not I restriction enzyme. The digested product was subcloned into the pBluescript II SK cloning vector (Stratagene) and sequenced. The sequence information was used in the generation of primer sequences which were subsequently used to PCR amplify the target gene encoding the endoglucanase. The primer sequences used were as follows:

5' primer: TTTATTCAATTGATTAAAGAGGAGAAATTA ACTATGATAAACGTTGC AACGGGAGAGGAG (SEQ ID NO:51) and  
3' primer: TTTATTGGATCCTACTTTGTGTCAACGAAGTATCC (SEQ ID NO:52).

The amplification product was digested with the restriction enzymes MfeI and BamHI. The digested product was then ligated to pQET cloning vector, a modified form of a pQE vector (Qiagen, Inc.) which was previously digested with BamHI and EcoRI compatible with MfeI. The pQE vector encodes antibiotic resistance (Amp<sup>r</sup>), a bacterial origin of replication (ori), an IPTG-regulatable promoter operator (P/O), a ribosome binding site (RBS), a 6-His tag and restriction enzyme sites.

The amplified sequences were inserted in frame with the sequence encoding for the RBS. The ligation mixture was then used to transform the *E. coli* strain M15/pREP4 (Qiagen, Inc.) by electroporation. M15/pREP4 contains multiple copies of the plasmid pREP4, which expresses the lacI repressor and also confers kanamycin resistance (Kan<sup>r</sup>). Positive recombinant

transformants were identified as having thermostable CMCase/endoglucanase activity by the assay described above. Plasmid DNA was isolated and confirmed by restriction analysis. Clones containing the desired constructs were grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml). The O/N culture was used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells were grown to an optical density 600 (O.D.<sup>600</sup>) of between 0.4 and 0.6. IPTG ("Isopropyl-B-D-thiogalacto pyranoside") was then added to a final concentration of 1 mM. IPTG induces by inactivating the lacI repressor, clearing the P/O leading to increased gene expression. Cells were grown an extra 3 to 4 hours. Cells were then harvested by centrifugation.

The primer sequences set out above may also be employed to isolate the target gene from the deposited material by hybridization techniques described above.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, within the scope of the appended claims, the invention may be practiced otherwise than as particularly described. It is to be understood that, while the invention has been described with reference to the above detailed description, the foregoing description is intended to illustrate, but not to limit, the scope of the invention. Other aspects, advantages, and modifications of the invention are within the scope of the following claims. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

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